AD-A204 381

DTIC FILE COPY

ᴀENTATION PAGE

Form Approved
OMB No 0704-0188

1a REPORT SECURITY CLASSIFICATION (U)	1b RESTRICTIVE MARKINGS NA
2a SECURITY CLASSIFICATION AUTHORITY NA	3 DISTRIBUTION/AVAILABILITY OF REPORT
2b DECLASSIFICATION DOWNGRADING SCHEDULE NA	Distribution Unlimited
4 PERFORMING ORGANIZATION REPORT NUMBER(S) NA	5 MONITORING ORGANIZATION REPORT NUMBER(S) NA

6a NAME OF PERFORMING ORGANIZATION EG&G Mason Research Institute	6b OFFICE SYMBOL (If applicable) NA	7a NAME OF MONITORING ORGANIZATION Office of Naval Research
6c ADDRESS (City, State, and ZIP Code) 57 Union Street Worcester, MA 01608		7b ADDRESS (City, State, and ZIP Code) 800 N. Quincy Street Arlington, VA 22217-5000

8a NAME OF FUNDING/SPONSORING ORGANIZATION Office of Naval Research	8b OFFICE SYMBOL (If applicable) ONR	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER N00014-88-6-0193

8c ADDRESS (City, State, and ZIP Code) 800 N. Quincy Street Arlington, VA 22217-5000	10 SOURCE OF FUNDING NUMBERS			

	PROGRAM ELEMENT NO	PROJECT NO	TASK NO	WORK UNIT ACCESSION NO
	61153N	RR04108		

11 TITLE (Include Security Classification)

(U) Surface-Bound Membrane-Mimetic Assemblies

12 PERSONAL AUTHOR(S)
Smith, H. Gilbert

13a TYPE OF REPORT Annual	13b TIME COVERED FROM 1/88 TO 10/88	14 DATE OF REPORT (Year, Month, Day) 88.10.31	15 PAGE COUNT 10

16 SUPPLEMENTARY NOTATION
NA

17	COSATI CODES		18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Biomembrane, Rhodopsin, Receptor Protein, Membrane-Mimetic, Immobilized Membrane, Biosensor
08			

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

This project aims to incorporate integral membrane proteins into matricies that mimic the natural lipid bilayer, but which are linked with electrically active solid surfaces. The structures are formed by adapting approaches that are commonly used to form model membrane vesicles in a manner that allows the membrane proteins and lipids to assemble into membrane structures on a surface. The objective is to use these structures to investigate stimulus-induced displacements of charges associated with membrane receptor proteins. The experimental focus is on charge movements in the photoreceptor protein, rhodopsin. The effort reported here has concentrated on characterizing the composition and biochemical properties of surface-bound membrane structures.

20 DISTRIBUTION/AVAILABILITY OF ABSTRACT ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	21 ABSTRACT SECURITY CLASSIFICATION (U)
22a NAME OF RESPONSIBLE INDIVIDUAL Dr. R.J. Nowak/Dr. I. Vodyanoy	22b TELEPHONE (Include Area Code) 202/696-4409	22c OFFICE SYMBOL ONR

DD Form 1473, JUN 86   *Previous editions are obsolete*   SECURITY CLASSIFICATION OF THIS PAGE

(U)

EG&G MASON RESEARCH INSTITUTE

57 Union Street   Worcester Massachusetts 01608   617 791 0931

31 October 1988

Annual Report
Contract N00014-88-C-0193

Principal Investigator: H. Gilbert Smith, Ph.D.

Contractor: EG&G Mason Research Institute

Title: Surface-Bound Membrane-Mimetic Assemblies:
Electrostatic Attributes of Integral Membrane Proteins

Research Objective

The objective of this project is to investigate stimulus-induced displacements of charges associated with integral membrane proteins. Membrane receptor proteins are incorporated into matrices that mimic the natural lipid bilayer and are bound to the surface of solid electrode materials. The focus of the investigation is on charge movements in the photoreceptor protein, rhodopsin.

Introduction

Membrane receptor proteins possess dynamic freedom within the constraints of the two dimensional lipid bilayer in which they reside. In order to form assemblies that constrain receptor proteins to a surface while retaining the natural motional freedom necessary for their function, we are investigating the formation of surface-bound structures resembling lipid bilayers. In concept, these structures represent protein-containing lipid bilayers linked to the surface through the lipid phase thereby leaving the proteins free to rotate and diffuse in the plane of the membrane just as in a natural membrane. Formation of these structures is driven by the natural self-assembly properties of membrane proteins and lipids. Work during the first phase of this project has focused on the formation and characterization of the surface-bound membrane structures.

88 11 10 014

Approach

Techniques that have been successful in forming functional free standing recombinant membranes are being adapted to form structures in which membrane proteins and lipids self-assemble onto solid surfaces. Briefly, a detergent dialysis procedure is used to reconstitute membrane components onto surfaces that have been made partially hydrophobic through covalent attachment of long-chained alkyl groups. The alkyl groups attached to the surface appear to provide an initiation point for membrane formation. The resultant structure is essentially a membrane immobilized through the lipid matrix. This project will use chemical, optical, biochemical, and electrochemical techniques to probe the structure of the surface-bound assemblies and to determine the effect of surface reconstitution on membrane receptor protein function.

The stimulus-induced conformational movements of membrane receptor proteins are accompanied by a redistribution of the protein's charged side chains with respect to the membrane surface. These movements cause changes in the electrical potential field at the membrane surface. Subsequent phases of this project will use the surface-bound assemblies to investigate charge movements that accompany membrane receptor function. These electrochemical studies will provide information on the dynamics of charge displacements in the membrane as well as additional characterization of the membrane structures themselves.

Rhodopsin, the protein in the retina that is responsible for low-light vision, has been chosen as the membrane receptor for this study because of its ready availability, ease of purification, proven ability to be reconstituted into functional membranes by detergent dialysis, and status as a representative integral membrane protein, particularly of that class of receptors that activate G-protein cascades. Rhodopsin responds to an easily controlled stimulus, light, and its chromophore, 11-cis retinal, provides a convenient, endogenous probe for controlling and monitoring protein functions and conformations. Furthermore, rhodopsin offers ample chemical and biochemical functions for testing the structural and functional viability of the proposed assemblies.

Outline of Method for Forming Membranes.

Preparation of Substrate. Most of the work to date has involved glass substrates including 37 μm diameter glass beads, microscope slides and cover slips. These are thoroughly cleaned and then reacted with octadecyltrichlorosilane for varying periods of time. The substrate is washed with organic solvents to remove unreacted reagent and then dried before the membrane deposition procedure. The alkylsilanated surfaces are noticeably hydrophobic after this process. We have

indirect evidence, based upon varying reaction conditions, that complete coverage of the surface with alkyl groups is not necessary for successful membrane formation, and in fact may not be desirable. We are investigating various approaches for characterizing the extent of surface coverage as described later in this report.

Membrane Deposition. The alkylsilanated substrate is placed in one compartment of a flow dialysis chamber with a detergent solubilized solution of membrane protein and lipid. The membranes used for most of these studies are the disc organelles isolated from the outer segments of retinal rod cells which in turn are prepared from frozen, dark-adapted bovine retinas. Typical initial conditions are disc membranes providing 1 mg/ml rhodopsin solubilized in 30 mM octylglucoside, 100 mM KCl, 20 mM HEPES, pH 7.5. Octylglucoside has been chosen as a well defined detergent in which many membrane proteins are reasonably stable. Its relatively high critical micelle concentration (~25 mM) facilitates removal by dialysis.

The flow system is arranged so that dialysis is against a flow of buffer (100 mM KCl, 20 mM HEPES). The rate of detergent removal can be controlled by the porosity of the dialysis tubing used, by the rate of external buffer flow, and by adding detergent to the flow side of the chamber. In some experiments, dialysis is against a decreasing concentration of detergent. The aim of the flow dialysis procedure is to precisely control the reduction of the free detergent concentration, particularly near the critical micelle concentration

Composition of Surface-Bound Membranes

We have confirmed and extended preliminary results indicating that the surface-bound membranes have approximately the same protein and lipid composition as the natural disk membranes. In order to have sufficient material for compositional analysis, we have used small diameter (37 μm) glass beads as substrates. After the flow dialysis proced e, the dialysis chamber contains membrane vesicles in addition to the surface-bound material. The free-floating vesicles are removed from surface-bound material by centrifugation through a density gradient.

The rhodopsin content of the surface-bound membrane-structures is conveniently measured by extracting the rhodopsin with detergent and measuring the change in absorbance at 500 nm upon light exposure (ΔA_{500}, figure 1). The ΔA_{500} provides a specific measure of the amount of intact, not photolyzed, rhodopsin in a sample. The fact that rhodopsin retains its native absorption spectrum demonstrates that the detergent dialysis procedures by which the structures are formed do not cause gross denaturation of the protein. Rhodopsin in the surface-bound membranes can be

compared with the original, native membranes by normalizing the 500 nm absorbances to the 280 nm absorbances which represent the total protein in the sample. For example in the experiment shown in figure 1, the A_{280}/A_{500} ratio for the original solubilized disc membranes (fig. 1B) was 2.4. The A_{280}/A_{500} ratio for the rhodopsin extracted from the glass beads (fig. 1A) was 2.6. Although there may be some slight denaturation, the change in spectral ratio is probably accounted for by light scattering artifacts that become important at the low absorbances of the latter sample.
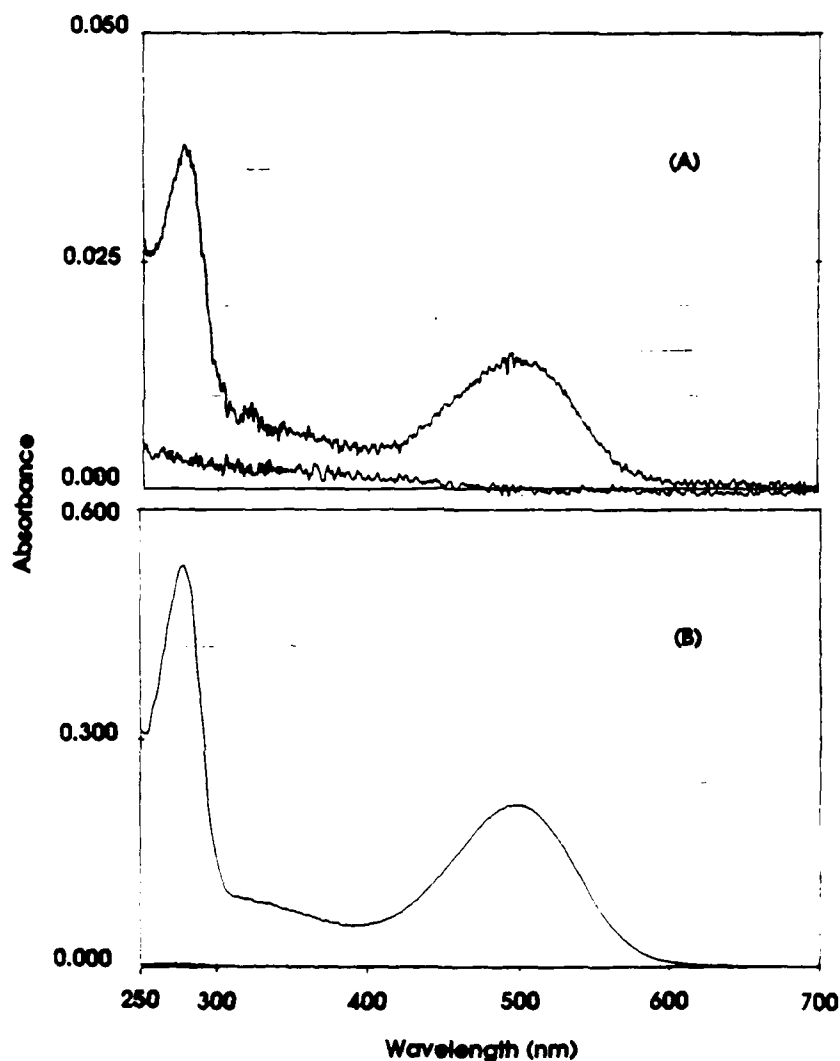

Figure 1. Comparison of Spectra of Solubilized Rhodopsin from
(A) Surface-Bound Structures and (B) Natural Membranes.

Results of several experiments measuring the composition of the surface-bound material are summarized in figure 2. From the measured amount of protein and the calculated surface area, we find an apparent spacing between rhodopsin molecules of 70 Å to 130 Å and a ratio of lipid to rhodopsin ranging from 60 to 240 mol lipid/mol rhodopsin. The samples that have the highest surface density of rhodopsin (smallest spacing between rhodopsin molecules) also have the lowest lipid/rhodopsin ratios suggesting that lipids fill in the extra space in samples having a lower protein content. In the natural disk membrane, the spacing between rhodopsin molecules is 50-60 Å with 60-100 lipids per rhodopsin.
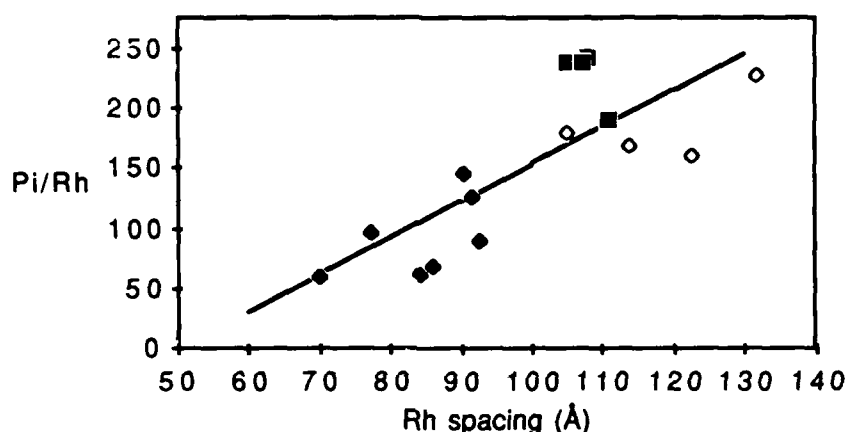

Figure 2. Rhodopsin and Lipid Content of Surface-Bound Membranes.

If the alkyl chains that are covalently attached to the surface were to completely replace the lipid in one leaflet of the bilayer, then the ratio of lipid to rhodopsin in the surface-bound membranes would be approximately one-half that of the natural membrane assuming the same surface density of rhodopsin as in the natural membrane. Based upon the fact that the smallest measured ratios of lipid to protein in the surface-bound assemblies are almost the same as those in the native membranes, we feel that the structures may better be described as lipid bilayers anchored to the surface by a few alkyl chains incorporated into one leaflet of the bilayer during membrane formation.

We tentatively attribute the variation in the incorporation of rhodopsin to the specific nature of the alkylsilanated substrate. In figure 2 the different symbols represent three modifications of the alkylsilanization process. The amount of protein that can be incorporated into the surface films might be expected to vary with the density of alkyl chains covalently attached to the surface. Conditions that are optimized for the alkylsilanization reaction may form an alkyl layer that does not leave "room" for protein incorporation. In experiments where we went to great

lengths to maximize the alkyl silanization reaction, by rigorously drying solvents, using fresh silane reagent and using freshly cleaned glass beads, we found less surface immobilized rhodopsin (fig.2, open diamonds). The optimal condition appears to be one where the substrate surface is made sufficiently hydrophobic to provide an initiation center for membrane formation, but not so fully packed with alkyl chains as to hinder protein insertion.

While we do not know the detailed molecular structure of the membrane assemblies, all of the evidence we have gathered to date suggests a uniform surface coverage consistent with a bilayer-type structure. As the surface density of rhodopsin decreases, the surface bound lipid increases as would be expected for constant surface coverage with varying amounts of incorporated protein. The change in surface properties of the glass beads upon detergent dialysis is also consistent with membrane deposition. There is a dramatic increase in surface hydrophobicity after alkylsilanization followed by the expected decrease in hydrophobicity after membrane deposition (the surface of the membrane is expected to be hydrophilic due to the polar lipid head groups). These changes in hydrophobicity can be observed visually from the contact angle of water droplets on the surface or with optical probes that are sensitive to the nature of the surface. These results, combined with the compositional data indicating that there is only sufficient material for a single bilayer on the surface, suggest that we may be obtaining structures that resemble natural biological membranes. Results of an ESCA analyses were also consistent with an extremely thin membrane structure.

This approach should also be applicable to other substrate materials that will react with silane reagents, including silicon oxide, silicon nitride, and oxide layers on metals such as platinum. We have preliminary results that indicate membrane formation on all of these substrates, but have obtained extensive compositional information only for structures formed on glass surfaces.

In order to demonstrate the applicability of this approach to proteins other than bovine rhodopsin, we have used analogous techniques with a second retinal-containing protein, bacteriorhodopsin. This protein is found in the purple membrane of *Halobacterium halobium* and serves as a light-driven proton pump to generate a transmembrane proton gradient used by the bacterium for ATP synthesis. The membranes are easily isolated from the bacteria and are remarkably stable. Like rhodopsin, this protein is also easily quantized and characterized by its absorption spectra. Figure 3 compares the spectra of bacteriorhodopsin in the natural purple membrane with that from protein solubilized from the surface-bound membrane. The absorption maxima of the protein is about 560 nm, and unlike rhodopsin it can be reversibly converted between its dark-adapted and light-adapted states. From the metastable light-adapted state there is a light-induced reaction cycle that underlies the proton pumping.
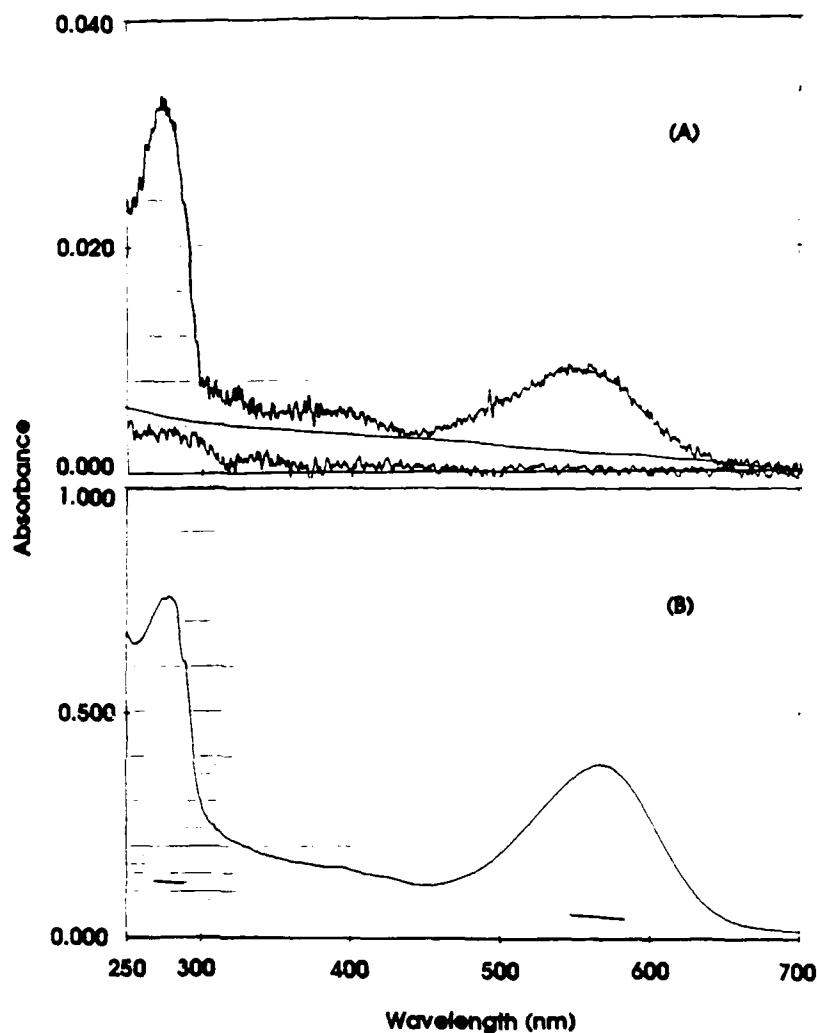
Figure 2. Comparison of Spectra of Bacteriorhodopsin:
(A) Solubilized from Surface-Bound Structures and (B) Natural Membranes

Biochemical Activity of Surface-Bound Membranes Containing Rhodopsin

In the rod photoreceptor disk membrane, rhodopsin acts as a light-activated trigger for a G-protein cascade that results in increased cGMP-phosphodiesterase activity within the rod cell cytoplasm. The system includes the integral membrane protein, rhodopsin, and two peripheral membrane enzymes – the G-protein or "transducin", and the phosphodiesterase. The peripheral proteins can be removed from the

natural membranes by treatment with low ionic strength buffers and added back to the "stripped" membranes to restore activity.

Rhodopsin's ability to activate the G-protein cascade upon light exposure offers a sensitive measure of the extent to which the functional properties of rhodopsin are preserved in the surface-bound membrane structures. The activity of the system is conveniently measured by a pH assay based upon protons released during cGMP hydrolysis. Although the assay conditions are not yet optimized, we have measured activities in suspensions of disk membranes from which the peripheral enzymes had been removed and subsequently restored. These reconstituted systems give about one-half the activity of isolated rod outer segments having the complete complement of endogenous enzymes. The reduced activity of the disks probably reflects the lower concentration of peripheral enzymes in the reconstitution assay as well as some denaturation of the peripheral enzymes. We have also obtained light-activated phosphodiesterase activity with the surface-bound membrane structures after adding peripheral enzymes. The activities measured to date are lower than for the reconstituted disk membrane system, and variable in magnitude. The results suggest that rhodopsin incorporated into the surface-bound membranes does retain its ability to activate the enzymatic cascade, but more work is needed to quantify the ratio of activity in the natural membrane to that in the surface-bound membranes. Factors that might cause reduced activity in the surface-bound membranes include rhodopsin not being oriented properly in the membranes, partial denaturation of some of the rhodopsin, and inhibition of rhodopsin's activity by interactions with the surface.

Characterization of Flow Dialysis Procedure

In membrane reconstitutions by detergent dialysis, the important concentration range is that near the critical micelle concentration (cmc, about 20-25 mM for octylglucoside). To obtain the data needed to precisely control the membrane deposition process, we have measured the octylglucoside concentrations in the effluent from the dialysis chamber using different profiles of the external detergent concentration and various types of dialysis membranes. Figure 3 shows data for the rate of removal of octylglucoside from the dialysis chamber using three types of dialysis membranes. The data are presented in terms of the octylglucoside concentration within the dialysis chamber. From the data summarized in Table I, it is apparent that the controlling factor in detergent removal is the type of dialysis membrane used. (The rate of detergent removal does not scale with the pore size (MWCO) because the dialysis membranes are made from different thickness materials.)
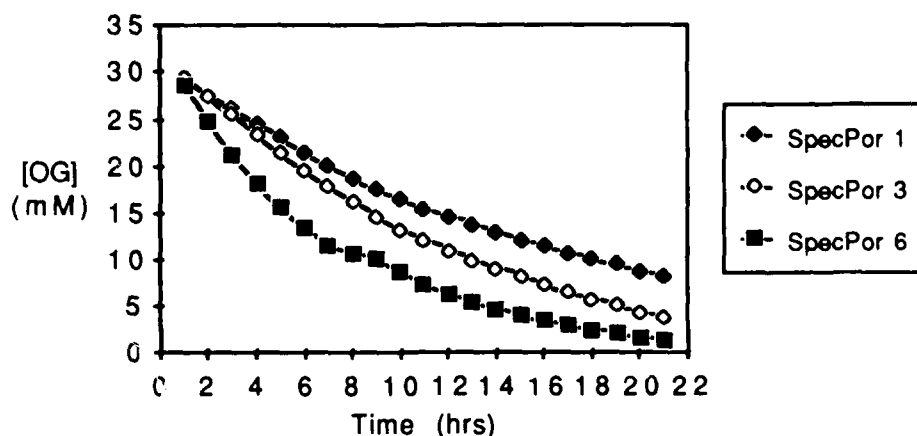
Figure 2. Detergent Removal by Flow Dialysis.

Table I. Rates of Octyl Glucoside Removal.

Type of Filter	Internal [OG] after 20 hr. run*	t1/2
SpectraPor 1 (6-8000 MWCO)	8.50 mM	11.00 hr.
SpectraPor 3 (3500 MWCO)	5.04 mM	6.93 hr.
SpectraPor 6 (1000 MWCO)	0.73 mM	4.80 hr.

* "Internal" refers to the side of the dialysis chamber in which the membrane material is placed (as opposed to the "external", flow side of the chamber).

Characterization of Alkylsilanated Surfaces

As the incorporation of rhodopsin into the surface-bound structures may vary with the packing density of alkyl chains on the surface, we are investigating ways to evaluate the alkylsilanization reactions of the substrates. Papers by Sagiv (JACS 102:92-98, 1980) suggested that this might be accomplished through the use of an amphiphilic dye such as 3,3'-dioctadecyloxacarbocyanine perchlorate (Molecular Probes, $diOC_{18}(3)$). Sagiv showed that "holes" could be made in alkylsilane layers by having the dye present during the alkylsilanization reaction. The dye competes with the alkylsilane compounds for surface sites during the reaction, but can be subsequently removed by treatment with strong solvents such as $CHCl_3$. Sagiv also used the partition of the dye into monolayer coatings on glass surfaces to identify "holes" in those coatings. The covalently attached monolayer was treated with a dye solution, washed with a weak solvent to remove excess, free dye, and then treated

with a $CHCl_3$ to remove the bound dye. The amount of bound dye, quantified by the fluorescence or absorbance of the $CHCl_3$ solution, provided a measure of the completeness to which the surface was covered by the monolayer

Our experiments confirmed the general outline of Sagiv's work, but we found that the end point of the wash needed to remove free dye was not sufficiently sharp to accurately measure bound dye inserted into the monolayer. It should also be noted that the size "hole" that would be probed by the dye (cross sectional area, about 170 $Å^2$) is much smaller than the "holes" needed for inserting protein (cross sectional area, about 500 $Å^2$).

A second approach is to measure the number of silanol groups remaining after a preparative alkylsilanization by labeling free silanol groups with radioactive tracers. The approach is to use a small, highly reactive silane coupling agent that can then be cross linked to a radioactively labeled molecule. Like the approach using the hydrophobic dyes, this requires that unreacted reagent be quantitatively removed to give reproducible, acceptably low backgrounds.

Conclusions

The results of the first phase of this study are consistent with the formation of surface-bound protein/lipid structures resembling natural bilayer membranes. The effort has concentrated on characterizing the composition and biochemical properties of the surface-bound membrane structures. We have explored variations on the detergent dialysis procedure used to form the structures and have examined approaches for evaluating the silanization reaction that forms the substrate. Additional data is being gathered in all of these areas with particular emphasis on the functional properties of receptor proteins in the surface-bound assemblies. Electrochemical investigations of assemblies formed on suitable substrates will initially be aimed at determining electrical properties of the membranes (resistance, capacitance, impedance, etc.). Investigations of the composition, structure and function of the surface-bound membrane assemblies will continue with special attention to correlations between the composition of the assemblies and the functional viability of rhodopsin.